

# Prasinoxanthin is absent in the green-colored dinoflagellate *Lepidodinium chlorophorum* strain NIES-1868: pigment composition and 18S rRNA phylogeny

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**Abstract** Green-colored plastids in the dinoflagellates *Lepidodinium chlorophorum* and *L. viride* have been widely believed as the remnant of an endosymbiotic prasinophyte. This hypothesis for the origin of the *Lepidodinium* plastids is solely based on an unpublished result quoted in Elbrächter and Schnepf (Phycologia 35:381–393, 1996) hinting at the presence of a characteristic carotenoid in prasinophytes, prasinoxanthin, in the *L. chlorophorum* cells. On the other hand, a recent work failed to detect prasinoxanthin in a culture of *L. chlorophorum*. Unfortunately, we cannot conduct any additional experiments to examine whether the two strains considered in the previous studies are truly of *L. chlorophorum*, as neither of the two strains is publicly available. We here investigated the pigment composition of *L. chlorophorum* strain NIES-1868 maintained as a mono-algal culture under laboratory conditions, and detected no sign of prasinoxanthin. The pigment composition of strain NIES-1868 is consistent with previous phylogenetic analyses based on plastid-encoded

genes of the same strain, which successfully excluded prasinoxanthin-containing algae from the origin of the *L. chlorophorum* plastid. We also determined nucleus-encoded 18S ribosomal RNA (rRNA) genes from four *Lepidodinium* strains (including strain NIES-1868). Analyses of 18S rRNA sequences showed an extremely close relationship among strain NIES-1868 and other *Lepidodinium* cells/strains originating from different geological locations, suggesting that the cells/strains corresponding to these rRNA sequences lack prasinoxanthin.

**Keywords** Plastid replacement · Tertiary endosymbiosis · Serial secondary endosymbiosis

## Introduction

The majority of photosynthetic dinoflagellates possess plastids containing chlorophylls (Chl) *a+c* and a unique carotenoid, peridinin (Taylor 1980). The peridinin-containing plastids are most likely derived from a red algal endosymbiont. However, some dinoflagellate lineages are known to bear non-canonical plastids lacking peridinin which are classified into seven types according to Moestrup and Daugbjerg (2007). Traditionally, pigment compositions have been used to infer the origin of the non-canonical plastids; for example, the non-canonical plastids containing 19'-hexanoyloxyfucoxanthin, fucoxanthin, and alloxanthin advocated the evolutionary connections to the plastids in haptophytes, diatoms, and cryptophytes, respectively (Withers and Haxo 1975; Withers et al. 1977; Suzuki and Ishimaru 1992; Meyer-Harms and Pollehn 1998; De Salas et al. 2003; Bergholtz et al. 2005; Tamura et al. 2005). The origins of these non-canonical plastids inferred from pigment compositions were confirmed

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by phylogenetic analyses of plastid-encoded genes (e.g., Takishita et al. 1999, 2002; Tengs et al. 2000; Horiguchi and Takano 2006). Importantly, the remote relationship amongst the dinoflagellate (host) lineages bearing non-canonical plastids indicates that the plastid replacement events took place in separate branches in dinoflagellate phylogeny (Saldarriaga et al. 2001; Shalchian-Tabrizi et al. 2006).

The genus *Lepidodinium* bears non-canonical plastids containing Chl-*a+b* (Watanabe et al. 1987, 1990), but their pigment composition has not been fully settled. *Lepidodinium* plastids have been stated to contain prasinoxanthin, a carotenoid exclusively found in a few members of the green algal class Prasinophyceae, implying that this dinoflagellate lineage acquired its current non-canonical plastids through the endosymbiosis of a prasinophyte (Elbrächter and Schnepf 1996). Nevertheless, the presence of prasinoxanthin in *L. chlorophorum* was quoted as a personal communication in Elbrächter and Schnepf (1996), and therefore, needs to be re-examined. To elucidate the precise origin of the *Lepidodinium* plastids, it would be ideal if the pigment composition, cell morphology, and gene sequences are available for the same strain maintained in the laboratory. Recently, Laza-Martinez et al. (2007) called the popular hypothesis for the origin of the *Lepidodinium* plastids into question, as no sign of prasinoxanthin was detected in the *L. chlorophorum* culture. Laza-Martinez et al. (2007) admitted that the *L. chlorophorum* culture examined was unfortunately not mono-algal, and they did not provide morphological or sequence data for the particular strain. Furthermore, to our knowledge, the prasinoxanthin-lacking '*L. chlorophorum*' strain has not been deposited in any culture collection.

Some of us have studied the evolutionary characteristics of the *L. chlorophorum* plastid by sequencing 11 plastid-encoded genes of *L. chlorophorum* strains (Takishita et al. 2008; Matsumoto et al. 2011a, b). The results showed that the plastids of the *L. chlorophorum* strains originated from one of "core chlorophytes," which did not contain prasinoxanthin. These strains have been maintained as mono-algal cultures in the laboratory, and their morphologies have been well characterized. Thus, these strains can be used to address the pigment composition of *L. chlorophorum*. In this study, we investigated the pigment composition of the *L. chlorophorum* strain NIES-1868, and revealed that this strain apparently lacked prasinoxanthin. In addition, we newly determined the nucleus-encoded 18S ribosomal RNA (rRNA) genes of strains NIES-1867 and NIES-1868, as well as those of two another *Lepidodinium* strains. The rRNA sequences from the four *Lepidodinium* strains determined in this study and four of those deposited under the name of

*L. chlorophorum* or *L. viride* in GenBank database shared nucleotide identity of  $\geq 99\%$ , and formed a tight clade in our phylogenetic analyses. Thus, the *Lepidodinium* species, of which 18S rRNA sequences bear high identities to that of strain NIES-1868, most likely lack prasinoxanthin.

## Materials and methods

### Algal cells

Two strains of *L. chlorophorum* (NIES-1867 and NIES-1868) were purchased from the Microbial Culture Collection at the National Institute for Environmental Studies (NIES; 16-2, Onogawa, Tsukuba, Ibaraki 305-8506, Japan). We considered the two strains to belong to identical species (or extremely closely related species) because (1) they originated from the exact same water sample collected in the East China Sea (see Takishita et al. 2008), (2) their cell morphologies were identical under the light microscope (data not shown), and (3) most gene sequences available for both strains were identical (see below). Additionally we examined two original clones of *Lepidodinium* strains: strain 'MH 360,' which was confirmed as *Lepidodinium* sp. by light microscopy, and strain 'T03-1,' which was identified as *L. viride* based on the presence of the body scales on the cell surface. Strain MH 360 was isolated from sea water collected offshore of the Sanriku region, Japan (Pacific Ocean), on September 9, 2009, while strain T03-1 was isolated from sea water collected offshore southeast of Phuket Island, Thailand (Andaman Sea), on

**Table 1** Nucleus-encoded 18S rRNA sequences of *Lepidodinium* species used in this study

	Strain	GenBank accession no.	Originally sampled at
<i>L. chlorophorum</i>	NIES-1868	AB686254	East China Sea
<i>L. chlorophorum</i>	NIES-1867	AB686253	East China Sea
<i>Lepidodinium</i> sp.	MH 360	AB686255	Pacific Ocean
<i>L. viride</i>	T03-1	AB686256	Andaman Sea
<i>L. viride</i>	MUCC247D <sup>a</sup>	AF022199	Unknown
<i>L. chlorophorum</i> <sup>b</sup>	Not available	AM184122	Unknown
<i>L. viride</i>	Not available	DQ499645	Unknown
<i>L. chlorophorum</i>	Not available	AY331681	Unknown
' <i>L. viride</i> '	Not available	JF791033	Gulf of Mexico <sup>c</sup>

<sup>a</sup> According to the strain name listed in the GenBank file, we assumed that this sequence originated from a strain deposited in the Massey University Culture Collection

<sup>b</sup> This strain was obtained from the Scandinavian Culture Collection of Algae and Protozoa (Shalchian-Tabrizi et al. 2006)

<sup>c</sup> Assumed from the title of the GenBank file

September 18, 2003. The four strains were cultured in MNK medium (Noël et al. 2004) at 20 °C under a 14-h light/10-h dark cycle.

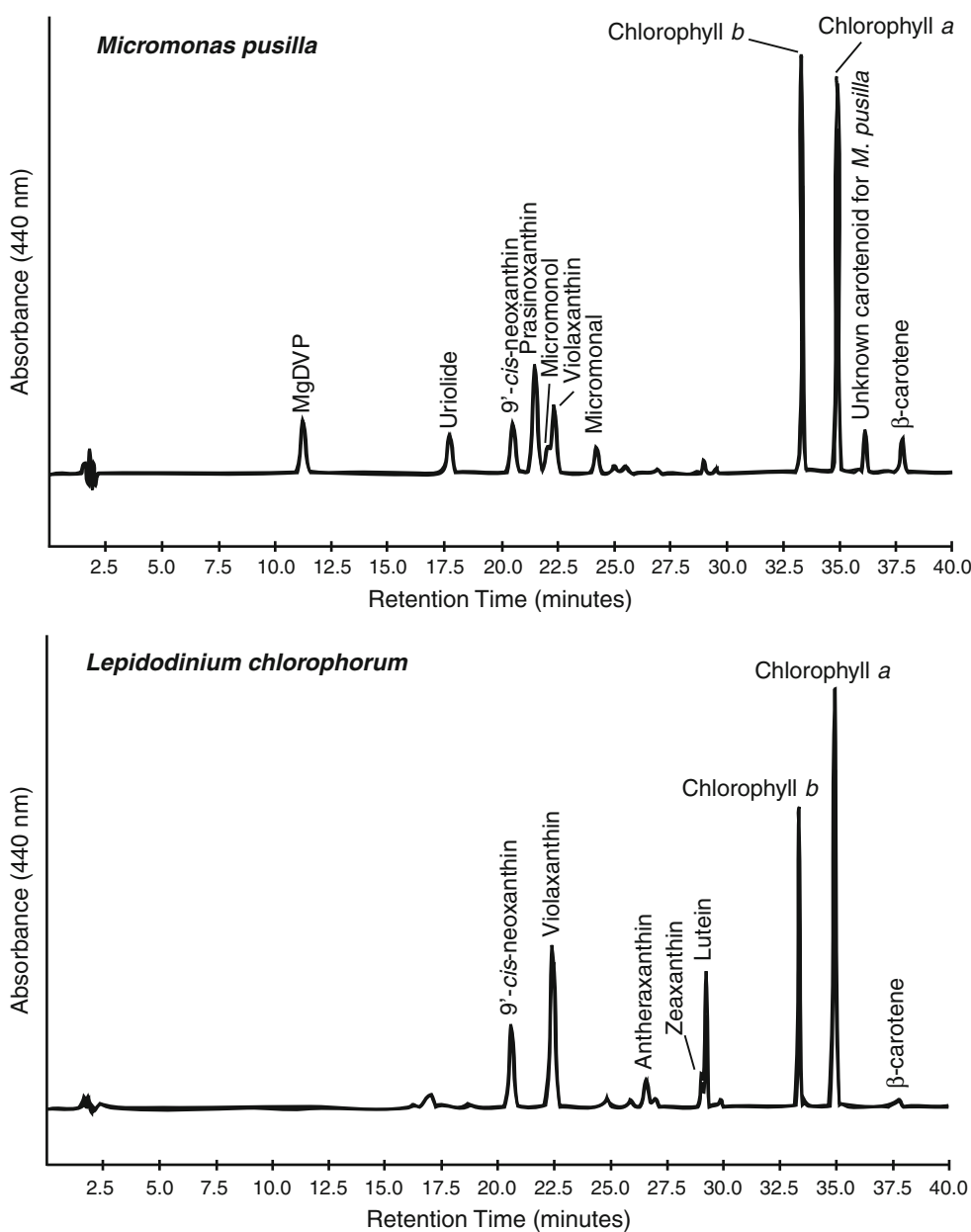
The prasinophycean green alga *Micromonas pusilla* (NIES-1411) was purchased from the Microbial Culture Collection at NIES and cultured in f/2 medium (Guillard and Rytner 1962) at 20 °C under a 14/10 h LD cycle.

**Pigment analysis**

*Lepidodinium chlorophorum* strain NIES-1868 and *M. pusilla* cells were harvested by centrifugation. After removal of a transparent viscosity layer on the cell pellet,

pigments were extracted with 100 µl of 100 % methanol, and the pigment extract was collected into a 1.5 ml centrifuge tube after centrifugation. We repeated the extraction until the extract was no longer colored. The extracted pigments were subjected to reverse-phase high-performance liquid chromatography (HPLC) with a Waters Symmetry C<sub>8</sub> column (150 mm × 4.6 mm; particle size 3.5 µm; pore size 100 Å). The HPLC was performed as described in Zapata et al. (2000) without any modifications. The eluted pigments were detected by the absorbance at 440 nm and identified by their elution patterns compared to those reported in Zapata et al. (2000).

**Fig. 1** HPLC chromatograms of pigments extracted from *Micromonas pusilla* NIES-1413 (top) and *Lepidodinium chlorophorum* NIES-1868 (bottom). Both algae were cultivated under uni-algal conditions. MgDVP, Mg-3,8-divinyl-pheoporphyrin a<sub>5</sub> monomethyl ester



### Preparation of nucleic acids, PCR, cloning, and sequencing

From the *Lepidodinium* cells, total DNA samples were extracted using the DNA Isolation Reagent (TaKaRa), and then, served to amplify nucleus-encoded 18S rRNA genes. We amplified 18S rRNA genes from total DNA by a pair of primers: 5'-GAAACTGCGAATGGCTCT-3' and 5'-CCGCAGGTTACCTAC-3'. The amplification conditions consisted of 30 cycles of 96 °C for 20 s, 55 °C for 20 s, and 72 °C for 3 min. Amplicons were cloned into pGEM T-Easy vector (Promega), and both strands were completely sequenced. The rRNA gene sequences determined in this study were deposited in DDBJ/EMBL/GenBank under the accession numbers AB686253–AB686256.

### Phylogenetic analysis

We retrieved nucleus-encoded 18S rRNA sequences of phylogenetically diverged dinoflagellates, including five sequences deposited as *Lepidodinium*, as well as closely related alveolates, from NCBI GenBank database. These retrieved sequences were then aligned with the four *Lepidodinium* sequences determined in this study. The details of *Lepidodinium* 18S rRNA sequences used in this study are listed in Table 1. The initial alignment was prepared by CLUSTALW ver. 1.83 (Thompson et al. 1994), and then

manually refined. After exclusion of all ambiguously aligned positions, the final 18S rRNA alignment included 105 taxa with 1,496 nucleotide positions.

The rRNA data set was subjected to the maximum-likelihood (ML) analysis under the GTR model incorporating among-site rate variation approximated by a discrete  $\Gamma$  distribution with four equally probable categories (GTR +  $\Gamma$  model) by using RAxML ver. 7.2.1 (Stamatakis 2006). The ML tree was heuristically searched from 10 distinct parsimony trees. A ML bootstrap analysis (100 replicates) was conducted under the GTR +  $\Gamma$  model with heuristic tree search from a single parsimony tree per replicate.

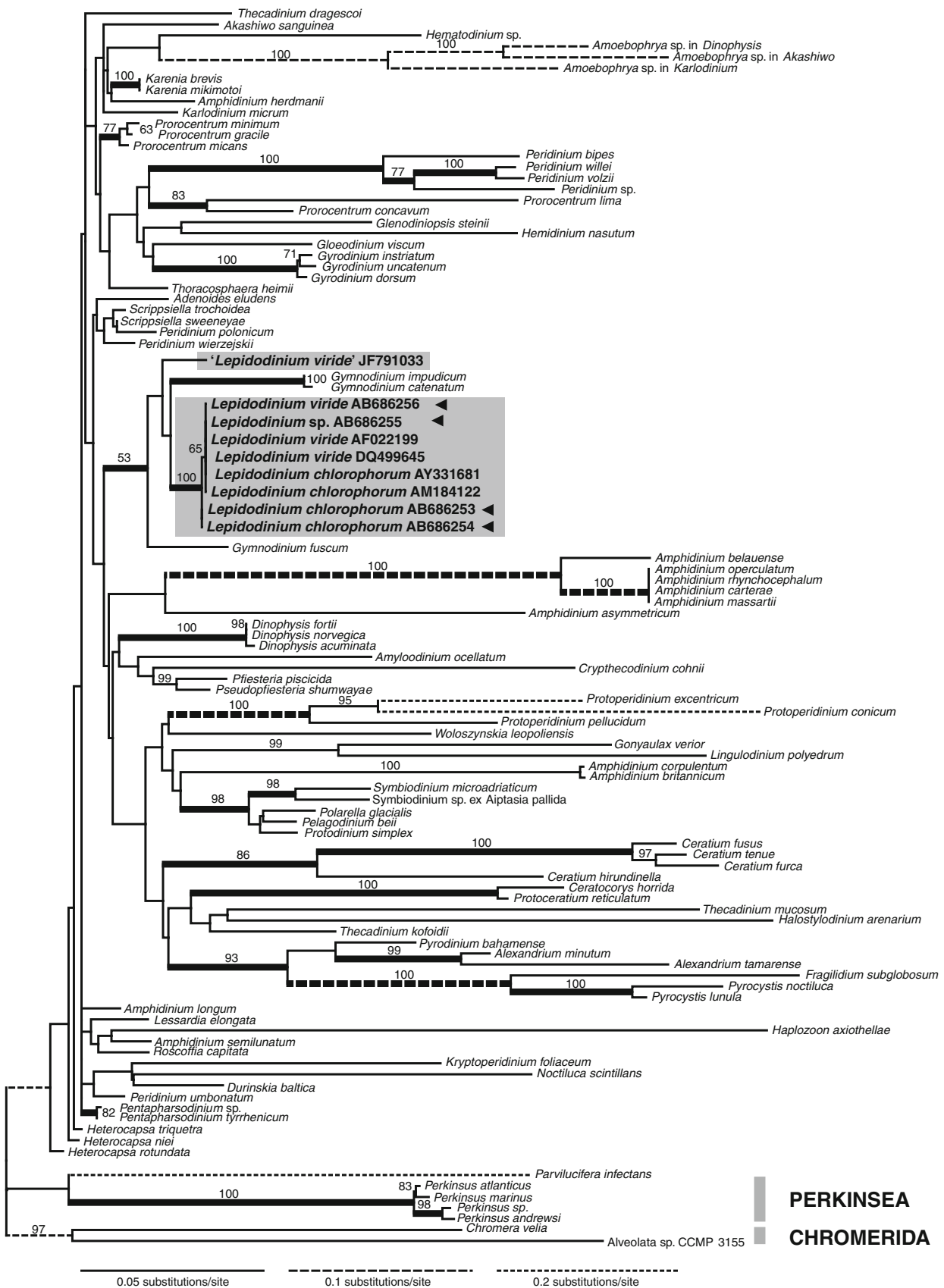
The same alignment was also subjected to Bayesian analysis with the GTR +  $\Gamma$  model by using MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003). Two parallel Markov chain Monte Carlo runs, each consisting of four incrementally heated chains, were run for  $10^6$  generations, sampling log-likelihoods and trees at 100-generation intervals. The first 20,000 generations were discarded as “burn-in,” and the majority consensus tree and Bayesian posterior probabilities were calculated from the remaining trees.

**Fig. 2** Maximum-likelihood phylogeny based on nucleus-encoded 18S rRNA sequences. Only bootstrap values  $\geq 50\%$  are presented. The bipartitions supported by  $\geq 0.95$  Bayesian posterior probabilities are highlighted by *thick lines*. The *Lepidodinium* sequences are *shaded*. The rRNA sequences determined in this study are highlighted by *arrowheads*

**Table 2** Pair-wise nucleotide identities among the nucleus-encoded 18S rRNA sequences from eight *Lepidodinium* species and *Gymnodinium catenatum*

	NIES-1867	MH 360	T03-1	AF022199	AM184122	DQ499645	AY331681	JF791033	<i>Gymnodinium catenatum</i>
<i>L. chlorophorum</i> NIES-1868	100 (0)	99.9 (1)	99.9 (1)	99.9 (1)	99.0 (16)	99.9 (1)	99.9 (1)	96.4 (59)	96.3 (61)
<i>L. chlorophorum</i> NIES-1867		99.9 (1)	99.9 (1)	99.9 (1)	99.0 (16)	99.9 (1)	99.9 (1)	96.4 (59)	96.3 (61)
<i>Lepidodinium</i> sp. MH 360			100 (0)	100 (0)	99.1 (15)	100 (0)	100 (0)	96.4 (60)	96.3 (62)
<i>L. viride</i> T03-1				100 (0)	99.1 (15)	100 (0)	100 (0)	96.4 (60)	96.3 (62)
<i>L. viride</i> AF022199					99.1 (15)	100 (0)	100 (0)	96.4 (60)	96.3 (62)
<i>L. chlorophorum</i> AM184122						99.1 (15)	99.1 (15)	95.5 (75)	95.4 (75)
<i>L. viride</i> DQ499645							100 (0)	96.4 (60)	96.3 (62)
<i>L. chlorophorum</i> AY331681								96.4 (60)	96.3 (62)
' <i>L. viride</i> ' JF791033									94.5 (91)

Mismatch positions out of 1,496 unambiguously aligned positions in the 18S rRNA alignment are given in parentheses





## Results

### Pigment composition

*Micromonas pusilla* is one of the standard algae containing prasinoxanthin (Zapata et al. 2000). As anticipated, our HPLC analysis of *M. pusilla* successfully identified prasinoxanthin, as well as Mg-3,8-divinyl-pheoporphyrin *a*<sub>5</sub> monomethyl ester (MgDVP), urolide, 9'-*cis*-neoxanthin, violaxanthin, micromonal, chlorophyll *b* (Chl *b*), chlorophyll *a* (Chl *a*),  $\beta$ -carotene, and an unknown carotenoid (Fig. 1, top). In contrast, in the same analysis of *L. chlorophorum* strain NIES-1868, 9'-*cis*-neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein, Chl *b*, Chl *a*, and  $\beta$ -carotene were detected, while prasinoxanthin was not found (Fig. 1, bottom). The overall pigment composition in strain NIES-1868 is of typical green algae, providing no clue for the origin of the *L. chlorophorum* plastid.

### 18S rRNA analysis

We newly determined 18S rRNA sequences from four *Lepidodinium* strains NIES-1867, NIES-1868, MH 360, and T03-1. The rRNA sequences from our strains determined here and the four sequences deposited in GenBank database—*L. viride* (AF022199), *L. viride* (DQ499645), *L. chlorophorum* (AY331681), and *L. chlorophorum* (AM184122)—appeared to be identical or almost identical (Table 2). In the 18S rRNA phylogeny, the eight *Lepidodinium* sequences tightly grouped together, and then, this *Lepidodinium* clade was placed within the dinoflagellate clade (Fig. 2). Interestingly, the rRNA sequence deposited as '*L. chlorophorum*' (JF791033) shared lower nucleotide identities with those of other *Lepidodinium* species (~96 %; Table 2), and it was excluded from the *Lepidodinium* clade (Fig. 2). Of note, *Gymnodinium catenatum*, instead of '*L. chlorophorum*' (JF791033), branched at the base of the *Lepidodinium* clade, although the relative position of JF791033 to other *Lepidodinium* sequences was inconclusive (Fig. 2).

## Discussion

We here investigated the pigment composition of *L. chlorophorum* strain NIES-1868, which is mono-algal culture maintained in the laboratory. Our results presented here indicate that *L. chlorophorum* strain NIES-1868 lacks prasinoxanthin, and the current Chl-*a+b*-containing plastid in this strain might be derived from a prasinoxanthin-lacking green alga. These data are consistent with the data presented in Laza-Martinez et al. (2007), suggesting no prasinoxanthin in their *L. chlorophorum* culture.

Importantly, the data on the pigment from strain NIES-1868 can be directly combined with the plastid-encoded gene sequences from the same strain determined in Matsumoto et al. (2011b). Indeed, the absence of prasinoxanthin is apparently consistent with a multigene phylogenetic analysis, which successfully excluded the evolutionary affinity between the plastid in strain NIES-1868 and any of those containing prasinoxanthin (Matsumoto et al. 2011b). Thus, we firmly conclude that (1) the *L. chlorophorum* strain NIES-1868 lacks prasinoxanthin and (2) the current Chl-*a+b*-containing plastid in this strain is derived from a prasinoxanthin-lacking green alga, as suggested in Matsumoto et al. (2011b).

The analyses of nucleus-encoded 18S rRNA sequences clearly indicated that the sequence of strain NIES-1868 was very closely related to those of the *Lepidodinium* cells sampled at various geographical locations. Thus, both pigment composition and phylogenetic data based on plastid-encoded genes from strain NIES-1868 discussed in the previous paragraph are most likely applicable to the cells corresponding to the rRNA sequences, which robustly grouped with the NIES-1868 sequence in the ML analysis. The rRNA sequence deposited as JF791033 in GenBank database, which was excluded from the *Lepidodinium* clade, may not have originated from a member of the genus *Lepidodinium*.

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